Title: METHODS TO DIAGNOSE A REQUIRED REGULATION OF TROPHOBLAST INVASION

Trophoblast Invasion

FIELD OF THE INVENTION

The invention relates to methods and compositions for diagnosing and treating conditions requiring regulation of trophoblast invasion.

BACKGROUND OF THE INVENTION

During placental development the establishment of fetal-maternal interactions is critical for a successful human pregnancy (1). Abnormalities of placenta formation due to shallow trophoblast invasion have been linked to preeclampsia and fetal growth restriction (2). In contrast, uncontrolled trophoblast invasion and abnormal trophoblast growth are associated with hydatiform mole and choriocarcinoma. In the course of placenta formation, chorionic villous cytotrophoblasts undergo two morphologically distinct pathways of differentiation. The vast majority of cytotrophoblasts in both floating and anchoring villi fuse to form the syncytiotrophoblast layer, which permits gas and nutrient exchange for the developing embryo. A small percentage of cytotrophoblasts in anchoring villi break through the syncytium, at selected sites, and generate columns of non-polarized cells which migrate into the endometrium. These extravillous trophoblasts (EVT) invade deeply into the uterus reaching the first third of the myometrium at which point they invade the spiral arteries, replacing their endothelium and vascular wall. Invasion peaks at 12 weeks of gestation and rapidly declines thereafter, indicating that, unlike tumour invasion, it is spatially and temporally regulated (3). Trophoblast invasion in the decidua is accompanied by a complex modulation of the synthesis and degradation of extracellular matrix (ECM) proteins and in the expression of adhesion molecules (4-6). Along the invasive pathway, ECM proteins undergo changes in their spatial distribution with loss of laminin and appearance of fibronectin (3,4). EVT loose the expression of E-cadherins, responsible for cell-cell adhesion between polarized stem cytotrophoblasts, down-regulate $\alpha_6\ \beta_4$ integrin, a laminin receptor, and acquire $\alpha_5\beta_1$ integrin, a fibronectin receptor (7). Once the EVT invade the endometrium they express the $\alpha_1\beta_1$ integrin, a collagen/laminin receptor. Thus, specific changes in ECM proteins and their receptors are associated with the acquisition of an invasive phenotype by the extravillous trophoblasts (4).

Preeclampsia occurs in 5-10% of pregnancies and is the leading cause of death and illness in women during pregnancy. Preeclampsia is also associated with considerable fetal/neonatal complications because of adverse intrauterine conditions and preterm delivery. There is currently no effective pharmacologic treatment for preeclampsia and the only remedy is to remove the placenta (and hence deliver the fetus preterm). Current protocols, including bedrest and antihypertensive drugs, seek to stabilize maternal/fetal condition until delivery is necessitated. It is estimated that around 200,000 children are born preterm in North America due to preeclampsia. Many of these babies will require costly intensive care at birth and if they survive may face a lifetime of chronic illness (e.g. lung disease) or disability (e.g. cerebral palsy, mental handicaps, blindness). These conditions represent a significant

impact on subsequent requirements for community health care resources. Therefore, reducing the incidence of preeclampsia and preterm birth would have a tremendous positive impact on health care delivery.

Summary of the Invention

The present inventors have studied the mechanisms that regulate trophoblast invasion. The inventors have found that antisense disruption of the expression of the TGFβ receptor, endoglin, triggers invasion of cytotrophoblast from first trimester villous explants *in vitro* indicating that the TGFβ receptor system, and in particular endoglin, plays a critical role in regulating this process. Significantly, the present inventors defined components that endogenously regulate trophoblast invasion. TGF-β_3 was found to be a major regulator of trophoblast invasion *in vitro*. In particular, the presence of TGF-β_3 and its receptors at 5-8 weeks at a time when there is no spontaneous trophoblast invasion and the absence of these molecules at 12-13 weeks when spontaneous invasion occurs, establishes a major role for TGF-β_3 as an endogenous inhibitor of trophoblast invasion. Down-regulation of TGF-β_3 (but not β_1 or β_2) expression using antisense oligonucleotides, stimulated extravillous trophoblast cell (EVT) outgrowth/migration and fibronectin production in 5-8 villous explants indicating that TGF-β_3 acts to suppress *in vivo* trophoblast invasion. The effects of antisense treatment to TGF-β_3 are specific as they are prevented by addition of exogenous TGF-β_3 but not TGF-β_1 or TGF-β_2. The stimulatory effects of TGF-β_3 are lost after 9 weeks of gestation which is compatible with TGF-β_3 being produced by the villi during a specific window of gestation within the first trimester (5-8 weeks) and that inhibition of its synthesis stimulates trophoblast differentiation. Addition of exogenous TGF-β_3 to the villous explants inhibits fibronectin synthesis.

The clinical importance of TGF-β_3 in regulating trophoblast invasion has been highlighted by the finding that TGF-β_3 is highly expressed in trophoblast tissue of preeclamptic patients when compared to that in age-matched control placenta while there was no change in the expression of either the β_1 or β_2 isoform. Fibronectin and α_5 integrin expression were also greater in preeclamptic placenta, indicating that in preeclampsia, where there is shallow trophoblast invasion, trophoblast cells are arrested as an α_5 integrin phenotype producing TGF-β_3. These data are supported by the finding that villous explants from a control (non-preeclamptic placenta, 32 weeks of gestation) spontaneously formed columns of trophoblasts that invaded the surrounding Matrigel, while explants from a preeclamptic placenta did not.

In contrast to TGF-β_3, activin, a TGF-β receptor, has been found to trigger trophoblast invasion. Follistatin an activin binding protein, inhibited the stimulatory effect of activin, and antibodies and antisense to endoglin.

Oxygen tension was also found to play a role in regulating trophoblast invasion. The expression of the hypoxia inducible factor, HIF-1α, parallels that of TGF-β_3 in first trimester trophoblast (i.e. peaks at 6-8 weeks but decreases after 9-10 weeks when oxygen tension increases). Expression of HIF-1α was dramatically increased in placentas of preeclamptic patients when compared to age-matched control tissue. Induction of HIF-1α by low PO_2 (around 6-8 weeks) up regulates TGF-β_3 transcription and blocks trophoblast invasion. A failure of the system to down-regulate at 9-11 weeks (either due to a block in response to normoxia or the absence of an increase in oxygen tension) leads to shallow invasion and predisposes to preeclampsia.

In addition to endoglin, the present inventors have found that TGF-β_3 signals through a receptor complex which includes RI (ALK1), RII and endoglin. While TGF-β RI (ALK-5) and TGF-β R-II are expressed throughout the villi and decidua at 9-10 weeks gestation, they were found to be absent from the base of the proximal columns of the anchoring villi at the transition zone between the villous and the invading EVT exactly at the site where endoglin is up-regulated. This dramatic change in TGF-β receptor expression indicates that EVT within the columns *in situ* are not subject to the inhibitory actions of TGFβ, but via R-I and R-II they come under the control of this ligand upon entering the decidua. In addition, antisense induced disruption of RI (ALK-1) and RII expression stimulated trophoblast outgrowth/migration and fibronectin synthesis. In contrast, antisense to RI (ALK-5) inhibited fibronectin synthesis.

Broadly stated the present invention relates to a method for detecting, preventing, and/or treating a condition requiring regulation of trophoblast invasion by modulating (a) TGFβ$_3$ (b) receptors of cytokines of the TGFβ family, (c) HIF-1α, and/or (d) O_2 tension. In accordance with one aspect of the invention a method is provided for diagnosing in a subject a condition requiring regulation of trophoblast invasion comprising detecting TGFβ$_3$, receptors of cytokines of the TGFβ family, or HIF-1α, in a sample from the subject. In an embodiment of the diagnostic method of the invention, a method is provided for diagnosing increased risk of preeclampsia in a subject comprising detecting TGFβ$_3$ or its receptors, or HIF-1α in a sample from the subject.

The invention also broadly contemplates a method for regulating trophoblast invasion comprising inhibiting or stimulating TGFβ$_3$, receptors of cytokines of the TGFβ family, HIF-1α, or O_2 tension. In an embodiment of the invention, a method is provided for increasing trophoblast invasion in a subject comprising administering to the subject an effective amount of an inhibitor of (a) TGFβ$_3$, (b) receptors of cytokines of the TGFβ family, and/or (c) HIF-1α. In a preferred embodiment of the invention a method is provided for treating a woman suffering from, or who may be susceptible to preeclampsia comprising administering therapeutically effective dosages of an inhibitor of (a) TGFβ$_3$, (b) receptors of cytokines of the TGFβ family, and/or (c) HIF-1α. A therapeutically effective dosage is an amount of an inhibitor of (a), (b) and/or (c) effective to down regulate or inhibit TGFβ$_3$ in the woman.

In another embodiment of the invention, a method is providing for reducing trophoblast invasion in a subject comprising administering an effective amount of (a) TGFβ$_3$; (b) receptors of cytokines of the TGFβ family; (c) HIF-1α; and/or (d) a stimulator of (a), (b) or (c). In a preferred embodiment, a method is provided for monitoring or treating choriocarcinoma or hydatiform mole in a subject comprising administering therapeutically effective dosages of (a) TGFβ$_3$; (b) receptors of cytokines of the TGFβ family; (c) HIF-1α; and/or (d) a stimulator of (a), (b) or (c). An amount is administered which is effective to up regulate or stimulate TGFβ$_3$ in the subject.

The invention also relates to a composition adapted for regulating trophoblast invasion comprising a substance which inhibits or stimulates TGFβ$_3$, receptors of cytokines of the TGFβ family, and/or HIF-1α, or regulates O_2 tension, in an amount effective to inhibit or stimulate trophoblast invasion, and an appropriate carrier, diluent, or excipient. In an embodiment of the invention, a composition is provided for treating a woman suffering from, or who may be susceptible to

preeclampsia, comprising a therapeutically effective amount of an inhibitor of (a) $TGF\beta_3$, (b) receptors of cytokines of the TGFβ family, and/or (c) HIF-1α, and a carrier, diluent, or excipient. In another embodiment of the invention, a composition is provided for monitoring or treating choriocarcinoma or hydatiform mole in a subject comprising a therapeutically effective amount of (a) $TGF\beta_3$; (b) receptors of cytokines of the TGFβ family; (c) HIF-1α; and/or (d) a stimulator of (a), (b) or (c), and a carrier, diluent, or excipient.

The invention further relates to a method of selecting a substance that regulates trophoblast invasion comprising assaying for a substance that inhibits or stimulates $TGF\beta_3$, receptors of a cytokine of the TGFβ family, or HIF-1α. The substances may be used in the methods of the invention to regulate trophoblast invasion.

The invention also relates to kits for carrying out the methods of the invention.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 shows the amino acid and nucleic acid sequence of $TGF\beta_3$;

Figure 2 shows the amino acid and nucleic acid sequence of HIF-1α;

Figure 3A are Southern blots showing expression of TGF-β isoforms in human placenta in the first trimester of gestation;

Figure 3B are photographs of immunoperoxidase staining of $TGF\beta_3$ performed in placental sections at 5, 8, and 12 weeks of gestation;

Figure 4A are photographs showing that addition of recombinant $TGF\beta_3$ to antisense $TGF\beta_3$ abolishes the antisense stimulatory effect on trophoblast budding and outgrowth;

Figure 4B are blots showing the reversal effect on antisense $TGF\beta_3$ stimulatory effect by exogenous $TGF\beta_3$ for fibronectin synthesis;

Figure 4C is a graph showing the changes in fibronectin estimated after normalization to control cultures;

Figure 4D are blots showing the effects on gelatinase activity in conditioned media of explants treated with sense or antisense oligonucleotides to $TGF\beta_3$;

Figure 4E are blots showing that the antisense $TGF\beta_3$ stimulatory effect on fibronectin production is lost after 9 weeks of gestation;

Figure 5A are blots showing message expression of TGFβ isoforms, α_5 integrin receptor, and fibronectin in preeclamptic and age-matched control placentae;

Figure 5B are photographs of immunoperoxidase staining of $TGF\beta_3$ performed in placental sections from normal pregnancies and pregnancies complicated by preeclampsia;

Figure 6A are photographs showing that antisense oligonucleotides to TGFβ$_3$ induces the formation of trophoblast cells in preeclamptic villous explants;

Figure 6B shows the results of gelatin Zymography of explants of 32 weeks gestation from preeclamptic placentae treated with antisense or control sense oligonucleotides to TGFβ$_3$ for 5 days;

Figure 6C are Western blots with MMP9 antisera of explants of 32 weeks gestation from preeclamptic placentae treated with antisense or control sense oligonucleotides to TGFβ$_3$ for 5 days;

Figure 7A is a blot showing expression of HIF-1α placenta in the first trimester of gestation;

Figure 7B is a blot showing expression of HIF-1α in preeclamptic (PE) and age-matched control placenta (C);

Figure 8 is a blot showing the effect of low oxygen tension on TGFβ$_3$ and HIF-1α expression in villous explants;

Figure 9 are photographs at 20% O_2 and 3% O_2 (25x and 50x) showing the effect of low oxygen tension on villous explant morphology; and

Figure 10 are photographs showing the effect of antisense to HIF-1α on villous explant morphology.

DETAILED DESCRIPTION OF THE INVENTION

1. Diagnostic Methods

As hereinbefore mentioned, the present invention provides a method for diagnosing in a subject a condition requiring regulation of trophoblast invasion comprising detecting TGFβ$_3$, receptors of cytokines of the TGFβ family, or HIF-1α in a sample from the subject. In an embodiment of the diagnostic method of the invention, a method is provided for diagnosing increased risk of preeclampsia in a subject comprising detecting TGFβ$_3$, its receptors, or HIF-1α in a sample from the subject.

TGFβ$_3$ is a cytokine of the TGFβ family and it has the structural characteristics of the members of the TGFβ family. TGFβ is produced as a precursor characterised by having an N-terminal hydrophobic signal sequence for translocation across the endoplasmic reticulum, a pro-region, and a C-terminal bioactive domain. Prior to release from the cell, the pro-region is cleaved at a site containing four basic amino acids immediately preceding the bioactive domain (Massague, 1990, Annu.Review. Cell Biol. 6:597).

The precursor structure of TGFβ is shared by members of the TGFβ family, with the exception of the TGFβ 4 precursor which lacks a distinguishable signal sequence. The degree of identity between family members in the C-terminal bioactive domain is from 25 to 90% (See Basler et al. Cell, 73:687, 1993, Figure 2). All nine cysteines are conserved in the bioactive domain in the TGFβ family. The bioactive domain is cleaved to generate a mature monomer.

The TGFβ family includes five members, termed TGFβ 1 through TGFβ 5, all of which form homodimers of about 25 kd (reviewed in Massague, 1990). The family also includes TGFβ 1.2 which is a heterodimer containing a β1 and a β2 subunit linked by disulfide bonds. The five TGFβ genes are highly conserved. The mature TGFβ processed cytokines produced from the members of the gene family show almost 100% amino acid identity between species, and the five peptides as a group show about 60-

80% identity. The amino acid sequence and nucleic acid sequence of $TGF\beta_3$ are shown in Figure 1 (See also sequences for GenBank Acession Nos. HSTGF31-HSTGF37, and HSTGFB3M).

"Receptors of cytokines of the TGFβ family" or "TGFβ receptors" refers to the specific cell surface receptors which bind to cytokines of the TGFβ family, in particular $TGF\beta_3$, including the TGF-β type I receptor (ALK-1 or ALK-5) (R-I), TGF-β type II receptor (R-II), betaglycan, endoglin and activin, and complexes of the receptors, in particular a RI-RII-endoglin complex. Endoglin binds $TGF\beta_1$ and β_3 with high affinity (K_D = 50pM). Betaglycan has considerable sequence homology to endoglin (Chiefetz, S., et al J. Biol Chem. 267: 19027, 1992; Lopez-Casillas, F., et al, Cell 67:785, 1991; Wang, X.F., et al, Cell 67:797, 1991), it can bind all three forms of $TGF\beta_3$, and it regulates access of the ligands to R-I and R-II which are serine/threonine kinases and unlike betaglycan, are necessary for signal transduction (Wrana, J.L. et al, Cell 71:1003, 1992, Lopez-Casillas et al, Cell 73:1435, 1993; Franzen, P., et al Cell 75:681, 1993; Laiho, M. et al, J. Biol. Chem. 266:9108; Massague, J. et al, Trends Cell Biol. 4:172, 1994). TGFβR-II is an integral membrane protein which contains a short extracellular domain, a single transmembrane domain, and an intracellular serine/threonine kinase domain (Lin H.Y. et al., Cell 68:775, 1992). Serine/threonine kinases encoding type II receptors have been cloned which are structurally related to the type II receptors (Wrana, J.L. et al, Cell 71:1003, 1992, ten Dikje, P., et al, Oncogene 8:2879, 1993; Ebner, R., et al Science 260:1344, 1993; Ebner, R., et al Science 262:900, 1993). TGFβ R-I (human ALK-5), binds $TGF\beta_1$ and β_3 only in the presence of TGFβ R-II (Wrana, J.L. et al, Cell 71:1003, 1992). The human ALK-1 (TGFβ R-I) binds TGFβ when forming a heterodimeric complex with TGFβ R-II (Franzen, P., et al Cell 75:681, 1993). TGFβ R-II kinase, which is endogenously phosphorylated, phosphorylates and activates R-I which then initiates further downstream signals (Wrana, J.L. et al, Nature 370:341, 1994).

Hypoxia-inducible factor-I (HIF-1) is present in nuclear extracts of many mammalian cells cultivated in a low oxygen atmosphere (Semenza, G.L. et al Mol. Cell. Biol. 12:5447, 1992; Wang, G.L. et al J. Biol. Chem. 268:21513, 1993). HIF-I binds as a phosphoprotein to a short DNA motif (BACGTSSK) identified in the 3'-flanking regions of many hypoxia-induced genes (Semenza, G.L. et al. J. Biol Chem 269:23757, 1994; Liu, Y., et al Circulation Res. 77:638, 1995; Firth, J.D. et al Proc. Natl. Acad. Sci. USA 91:6496, 1994; Abe, M., et al, Anal. Biochem. 216:276, 1994). HIF-I binds DNA as a heterodimeric complex composed of two subunits of the inducible HIF-1α and the constitutively expressed HIF -Iβ.

$TGF\beta_3$, receptors of cytokines of the TGFβ family (e.g.TGFβ RI (ALK-1), TGFβ RII, or a complex of RI-RII-endoglin), or HIF-1α may be detected in a variety of samples from a patient. Examples of suitable samples include cells (e.g. fetal or maternal); and, fluids (fetal or maternal), including for example, serum, plasma, amniotic fluid, saliva, and conditioned medium from fetal or maternal cells.

$TGF\beta_3$, receptors of cytokines of the TGFβ family, or HIF-1α may be detected using a substance which directly or indirectly interacts with the cytokine, TGFβ receptors, or HIF-1α. For example, antibodies specific for $TGF\beta_3$, the TGFβ receptors, or HIF-1α may be used to diagnose and monitor a condition requiring regulation of trophoblast invasion. A method of the invention using

antibodies may utilize Countercurrent Immuno-Electrophoresis (CIEP), Radioimmunoassays, Radioimmunoprecipitations, and Enzyme-Linked Immuno-Sorbent Assays (ELISA), Dot Blot assays, Inhibition or Competition assays and sandwich assays (see U.S. Patent Nos. 4,376,110 and 4,486,530).

Antibodies used in the methods of the invention include monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, and $F(ab')_2$ and recombinantly produced binding partners. Polyclonal antibodies may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, various fowl, rabbits, mice, or rats. Monoclonal antibodies may also be readily generated using conventional techniques (see U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993 which are incorporated herein by reference; see also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference). Binding partners may be constructed utilizing recombinant DNA techniques to incorporate the variable regions of a gene which encodes a specifically binding antibody (See Bird et al., Science 242:423-426, 1988).

Antibodies may also be obtained from commercial sources. For example, antibodies to $TGF\beta_3$ may be obtained from American Diagnostics Inc., CT, USA, Oncogene Science, NY, USA, and Dimension Laboratories, Mississauga, Canada.

The presence of $TGF\beta_3$ in a sample may also be determined by measuring the binding of the cytokine to compounds which are known to interact with $TGF\beta_3$ such as its receptors, or decorin, thrombospondin, the serum glycoprotein α2-macroglobulin, fetuin, or thyroglobulin (Y. Yamaguchi, D. M. Mann, E. Ruoslahti, Nature 346, 281 (1990); S. Scholtz-Cherry J.E. Murphy-Ullrich, *J. Cell Biol.* 122, 923 (1993); O'Conner-McCourt, L.M. Wakefield *J. Biol. Chem.* 262, 14090 (1987); and J. Massague *Curr. Biol.* 1, 117 (1991)). These compounds are referred to herein as "TGFβ Binding Compounds".

The presence of receptors of cytokines of the TGFβ family may be determined by measuring the binding of the receptors to molecules (or parts thereof) which are known to interact with the receptors such as their ligands. In particular, peptides derived from sites on ligands which bind to the receptors may be used. A peptide derived from a specific site on a ligand may encompass the amino acid sequence of a naturally occurring binding site, any portion of that binding site, or other molecular entity that functions to bind an associated molecule. A peptide derived from such a site will interact directly or indirectly with an associated receptor molecule in such a way as to mimic the native binding site. Such peptides may include competitive inhibitors, enhancers, peptide mimetics, and the like as discussed below.

The presence of HIF-1α may be determined by measuring the binding of HIF-α1 to DNA molecules which are known to interact with HIF-α1 such as hypoxia inducing genes. The TGFβ binding compounds and molecules that interact with the receptors and HIF-1α are referred to herein as "Binding Compounds".

The antibodies specific for the $TGF\beta_3$, TGFβ receptors, or HIF-1α, or the Binding Compounds may be labelled using conventional methods with various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase,

alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive materials include radioactive phosphorous ^{32}P, iodine I^{125}, I^{131} or tritium.

An antibody to $TGF\beta_3$, a TGFβ family receptor, or HIF-1α, or a Binding Compound may also be indirectly labelled with a ligand binding partner. For example, the antibodies, or a $TGF\beta_3$ Binding Compound may be conjugated to one partner of a ligand binding pair, and the $TGF\beta_3$ may be coupled to the other partner of the ligand binding pair. Representative examples include avidin-biotin, and riboflavin-riboflavin binding protein. Preferably the antibodies are biotinylated. Methods for conjugating the antibodies discussed above with the ligand binding partner may be readily accomplished by one of ordinary skill in the art (see Wilchek and Bayer, "The Avidin-Biotin Complex in Bioanalytical Applications,"AnaL Biochem. 171:1-32, 1988).

The antibodies or Binding Compounds used in the method of the invention may be insolubilized. For example, the antibodies or Binding Compounds may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc. The insolubilized compound or antibodies may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

Indirect methods may also be employed in which a primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against the cytokine. By way of example, if the antibody having specificity against $TGF\beta_3$ is a rabbit IgG antibody, the second antibody may be goat anti-rabbit gamma-globulin labelled with a detectable substance as described herein.

$TGF\beta_3$, TGFβ receptors, or HIF-1α can also be assayed in a sample using nucleotide probes to detect nucleic acid molecules encoding a $TGF\beta_3$, the TGFβ receptors, or HIF-1α. Suitable probes include nucleic acid molecules based on nucleic acid sequences encoding $TGF\beta_3$, the TGFβ receptors, or HIF-1α. A nucleotide probe may be labelled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as ^{32}P, ^{3}H, ^{14}C or the like. Other detectable substances which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and luminescent compounds. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization. Labelled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.).

A nucleic acid molecule encoding $TGF\beta_3$, TGFβ receptors, or HIF1α can also be detected by selective amplification of the nucleic acid molecules using polymerase chain reaction (PCR) methods.

Synthetic oligonucleotide primers can be constructed from the sequences of the TGFβ$_3$, TGFβ receptors, or HIF1α using conventional methods. A nucleic acid can be amplified in a sample using these oligonucleotide primers and standard PCR amplification techniques.

In a preferred embodiment of the invention, a method is provided for diagnosing increased risk of preeclampsia in a subject comprising detecting TGF-β$_3$, TGFβ R-I (ALK-1), TGFβ R-II, endoglin, HIF-1 α, or a complex of R-I (ALK-1)-R-II-endoglin in a sample, and in particular using antibodies specific for TGF-β$_3$. Levels of TGF-β$_3$, TGFβ receptors or complexes thereof, or HIF-1α may be measured during the first trimester of pregnancy (approximately 1 to 14 weeks). It is preferred that at least two measurements be taken during this period, preferably including a measurement at about 14 to 16 weeks. If the levels are significantly increased as compared to levels typical for women who do not suffer from preeclampsia, the patient is diagnosed as having an increased risk of suffering preeclampsia. Levels above those typical for women who do not suffer from preeclampsia may be suspect and further monitoring and measurement of TGFβ$_3$, TGFβ receptors, or HIF-1α may be appropriate. The information from the diagnostic method may be used to identify subjects who may benefit from a course of treatment, such as treatment via administration of inhibitors of TGFβ, as discussed herein.

It will also be appreciated that the above methods may also be useful in the diagnosis or monitoring of choriocarcinoma or hydatiform mole which involves uncontrolled trophoblast invasion (i.e. may be associated with abnormally low levels of TGFβ$_3$, TGFβ family receptors, or HIF1α). Further the above methods may be used to diagnose or monitor other pregnancy complications including intrauterine growth restriction, molar pregnancy, preterm labour, preterm birth, fetal anomalies, and placental abruption. The diagnostic and monitoring methods of the invention may also involve determining responsiveness of cells to oxygen.

The invention also relates to kits for carrying out the methods of the invention. The kits comprise instructions, negative and positive controls, and means for direct or indirect measurement of TGFβ$_3$, TGFβ receptors, or HIF1α.

2. Regulation of Trophoblast Invasion in a Subject

The invention also provides a method of regulating trophoblast invasion comprising directly or indirectly inhibiting or stimulating (a) TGFβ$_3$, (b) receptors of cytokines of the TGFβ family, (c) HIF1α; and/or (d) O_2 tension, preferably inhibiting or stimulating TGF-β$_3$. Trophoblast invasion may also be regulated by optimizing oxygenation of tissues.

In an embodiment of the invention, a method is provided for increasing trophoblast invasion in a subject comprising administering an effective amount of a substance which is an inhibitor of (a) TGF-β$_3$, (b) receptors of cytokines of the TGFβ family, and/or (c) HIF-1α. In particular, methods are provided for treating a women suffering from or who may be susceptible to preeclampsia.

In another embodiment of the invention, a method is providing for reducing trophoblast invasion in a subject comprising administering an effective amount of (a) TGFβ$_3$; (b) receptors of cytokines of the TGFβ family; (c) HIF-α1; and/or (d) a stimulator of (a), (b) or (c). The method may be used to monitor or treat choriocarcinoma or hydatiform mole.

The methods of the invention may also be used to monitor or treat other complications of pregnancy such as intrauterine growth restriction, molar pregnancy, preterm labour, preterm birth, fetal anomalies, or placental abruption.

Substances that regulate trophoblast invasion can be selected by assaying for a substance that inhibits or stimulates the activity of TGF-β_3, TGFβ receptors, or HIF-1α. A substance that regulates trophoblast invasion can also be identified based on its ability to specifically interfere or stimulate the interaction of (a) TGF-β_3 and a receptor for the cytokine (e.g. the interaction of TGFβ_3 and endoglin, or TGF-β_3 and R-I, R-II, or a complex of R-I-R-II endoglin, or (b) TGF-β_3 and HIF1α.

Therefore, a method is provided for evaluating a compound for its ability to regulate trophoblast invasion comprising the steps of:

(a) reacting TGFβ_3 or a part thereof that binds to a receptor of a cytokine of the TGFβ family, with a receptor of a cytokine of the TGFβ family or a part thereof that binds to TGFβ_3, and a test substance, wherein the TGFβ_3 and receptor of a cytokine of the TGFβ family or parts thereof, are selected so that they bind to form a ligand-receptor complex; and

(b) comparing to a control in the absence of the substance to determine the effect of the substance.

In particular, a method is provided for identifying a substance which regulates trophoblast invasion comprising the steps of:

(a) reacting TGFβ_3 or a part thereof that binds to a receptor of a cytokine of the TGFβ family, and a receptor of a cytokine of the TGFβ family or a part thereof that binds to TGFβ_3, and a test substance, wherein the TGFβ_3 and receptor of a cytokine of the TGFβ family or parts therof, are selected so that they bind to form a ligand-receptor complex, under conditions which permit the formation of ligand-receptor complexes, and

(b) assaying for complexes, for free substance, for non-complexed TGFβ_3 or receptor, or for activation of the receptor.

The substance may stimulate or inhibit the interaction of TGFβ or a part thereof that binds the TGFβ receptor, and the TGFβ receptor.

In an embodiment of the invention a receptor complex is employed comprising TGFβ R-I (ALK-1)-TGFβ RII-endoglin.

Activation of the receptor may be assayed by measuring phosphorylation of the receptor, or by assaying for a biological affect on a cell, such measuring biochemical markers of trophoblast invasion such as cell proliferation, FN synthesis, integrin expression, up regulation of gelatinase and type IV collagenase expression and activity.

The invention also provides a method for evaluating a substance for its ability to regulate trophoblast invasion comprising the steps of:

(a) reacting TGFβ_3 or a part of TGFβ_3 that binds to HIF-1α, HIF-1α or a part of the protein that binds to TGFβ_3, and a test substance, wherein the TGFβ_3 or part thereof, and HIF-1α or part thereof bind to form a TGFβ3-HIF-1α complex; and

(b) comparing to a control in the absence of the substance to determine the effect of the substance.

The substance may stimulate or inhibit the interaction of $TGF\beta_3$ and HIF-1α, or the activation of TGFβ by HIF-1α and thereby regulate trophoblast invasion.

The substances identified using the methods of the invention include but are not limited to peptides such as soluble peptides including Ig-tailed fusion peptides, members of random peptide libraries and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids, phosphopeptides (including members of random or partially degenerate, directed phosphopeptide libraries), antibodies [e.g. polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, single chain antibodies, fragments, (e.g. Fab, $F(ab)_2$, and Fab expression library fragments, and epitope-binding fragments thereof)], and small organic or inorganic molecules. The substance may be an endogenous physiological compound or it may be a natural or synthetic compound. The substance may be a TGFβ R-I-TGFβ R-II-endoglin complex, which competitively inhibits the binding of $TGF\beta_3$ to its natural receptors. The invention contemplates isolated TGFβ R-I-TGFβ R-II-endoglin complexes and their use in regulating trophoblast invasion.

The substances may be peptides derived from the binding sites of TGF-β_3 and a receptor for the cytokine such as endoglin, R-I or R-II, or a complex of R-I-R-II-endoglin; or the binding sites of TGF-β_3 and HIF1α. A peptide derived from a specific binding site may encompass the amino acid sequence of a naturally occurring binding site, any portion of that binding site, or other molecular entity that functions to bind an associated molecule. A peptide derived from such a binding site will interact directly or indirectly with an associated molecule in such a way as to mimic the native binding domain. Such peptides may include competitive inhibitors, enhancers, peptide mimetics, and the like. All of these peptides as well as molecules substantially homologous, complementary or otherwise functionally or structurally equivalent to these peptides may be used for purposes of the present invention.

"Peptide mimetics" are structures which serve as substitutes for peptides in interactions between molecules (See Morgan et al (1989), Ann. Reports Med. Chem. 24:243-252 for a review). Peptide mimetics include synthetic structures which may or may not contain amino acids and/or peptide bonds but retain the structural and functional features of a peptide, or enhancer or inhibitor of the invention. Peptide mimetics also include peptoids, oligopeptoids (Simon et al (1972) Proc. Natl. Acad. Sci USA 89:9367); and peptide libraries containing peptides of a designed length representing all possible sequences of amino acids corresponding to a peptide of the invention.

Peptides may be synthesized by conventional techniques. For example, the peptides may be synthesized by chemical synthesis using solid phase peptide synthesis. These methods employ either solid or solution phase synthesis methods (see for example, J. M. Stewart, and J.D. Young, Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Co., Rockford III. (1984) and G. Barany and R.B. Merrifield, The Peptides: Analysis Synthesis, Biology editors E. Gross and J. Meienhofer Vol. 2 Academic Press, New York, 1980, pp. 3-254 for solid phase synthesis techniques; and M Bodansky, Principles of Peptide Synthesis, Springer-Verlag, Berlin 1984, and E. Gross and J. Meienhofer, Eds., The Peptides: Analysis, Synthesis, Biology, supra, Vol 1, for classical solution synthesis.)

Peptide mimetics may be designed based on information obtained by systematic replacement of L-amino acids by D-amino acids, replacement of side chains with groups having different electronic properties, and by systematic replacement of peptide bonds with amide bond replacements. Local conformational constraints can also be introduced to determine conformational requirements for activity of a candidate peptide mimetic. The mimetics may include isosteric amide bonds, or D-amino acids to stabilize or promote reverse turn conformations and to help stabilize the molecule. Cyclic amino acid analogues may be used to constrain amino acid residues to particular conformational states. The mimetics can also include mimics of inhibitor peptide secondary structures. These structures can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins. Peptoids may also be used which are oligomers of N-substituted amino acids and can be used as motifs for the generation of chemically diverse libraries of novel molecules.

A substance that regulates trophoblast invasion may be a molecule which interferes with the transcription and/or translation of $TGF\beta_3$, TGFβ receptors, or HIF-1α. For example, the sequence of a nucleic acid molecule encoding $TGF\beta_3$, TGFβ receptors (e.g. endoglin, R-I (ALK-1), R-II, or RI-RII-endoglin complex), or fragments thereof, may be inverted relative to its normal presentation for transcription to produce an antisense nucleic acid molecule. An antisense nucleic acid molecule may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. Examples of antisense molecules for $TGF\beta_3$ are 5'-CCTTTGCAAGTGCATC-3' and 5'-GATGCACTTGCAAAGG-3'.

The treatment methods and compositions described herein may use substances that are known inhibitors of $TGF\beta_3$. For example, antibodies to $TGF\beta_3$, the TGFβ Binding Compounds including decorin, α2-macroglobulin, fetuin, and thyroglobulin, or peptides derived from the sites on these compounds that bind to $TGF\beta_3$, or chimeras of these molecules may be employed.

Activin, another member of the TGFβ receptor family, triggers trophoblast invasion and therefore it may be used to enhance trophoblast invasion in a subject.

The utility of a selected inhibitor or stimulator may be confirmed in experimental model systems. For example, the human villous explant culture system described by Genbacev et al. (21) can be used to confirm the utility of an inhibitor for treatment of preeclampsia.

In a preferred embodiment of the invention a method is provided for treating a woman suffering from, or who may be suspectible to preeclampsia comprising administering therapeutically effective dosages of an inhibitor of $TGF\text{-}\beta_3$ or TGFβ receptors, an inhibitor of HIF-1α, or a substance identified in accordance with the methods of the invention. Preferably treatment with the inhibitor begins early in the first trimester, at about 10 to about 16 weeks, and may continue until measured $TGF\text{-}\beta_3$ levels, TGF-β receptor levels, or HIF-1α levels are within the normal range. Preferably, treatment with the inhibitor or substance is not continued beyond about 30 weeks of gestation. For the purposes of the present invention normal $TGF\text{-}\beta_3$ levels, TGFβ receptor levels, or HIF-1α levels are defined as those levels typical for pregnant women who do not suffer from preeclampsia. Treatment with the inhibitor is discontinued after $TGF\text{-}\beta_3$ levels, TGF-β receptor levels, and/or HIF-1α levels are within normal range, and before any adverse effects of administration of the inhibitor are observed.

One or more inhibitors or one or more stimulators of $TGF\beta_3$, TGFβ receptors, or HIF-1α, or substances selected in accordance with the methods of the invention including Binding Compounds, may be incorporated into a composition adapted for regulating trophoblast invasion. In an embodiment of the invention, a composition is provided for treating a woman suffering from, or who may be susceptible to preeclampsia, comprising a therapeutically effective amount of an inhibitor of $TGF\beta_3$, TGFβ receptors, or HIF-1α, or substance selected in accordance with the methods of the invention including TGFβ Binding Compounds, and a carrier, diluent, or excipient.

The compositions of the invention contain at least one inhibitor or stimulator of $TGF\text{-}\beta_3$, TGFβ receptors, or HIF-1α, or substance identified in accordance with the methods of the invention, alone or together with other active substances. Such compositions can be for oral, parenteral, or local use. They are therefore in solid or semisolid form, for example pills, tablets, and capsules.

The composition of the invention can be intended for administration to subjects such as humans or animals. The pharmaceutical compositions can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle, carrier or diluent. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985).

The compositions of the invention may be administered together with or prior to administration of other biological factors that have been found to affect trophoblast proliferation. Examples of these factors include IL-11 (Ireland et al Blood 84:267a, 1994), G-CSF, GM-CSF and M-CSF (U.S. 5,580,554 to Keith).

The compositions and other biological factors may be administered through any known means. Systemic administration, such as intravenous or subcutaneous administration is preferred. A therapeutically effective amount of an active ingredient e.g. inhibitor is an amount effective to elicit the desired therapeutic response but insufficient to cause a toxic reaction. The dosage for the compositions is determined by the attending physician taking into account factors such as the condition, body weight, diet of the subject, and the time of administration.

For example, a therapeutically effective dose of an inhibitor, e.g. an amount sufficient to lower levels of $TGF\beta_3$ to normal levels, is about 1 to 200 μg/kg/day. The method of the invention may involve a series of administrations of the composition. Such a series may take place over a period of 7 to about 21 days and one or more series may be administered. The composition may be administered initially at the low end of the dosage range and the dose will be increased incrementally over a preselected time course.

An inhibitor or stimulator of $TGF\beta_3$, receptors of cytokines of the TGFβ family, or HIF-1α, or substance identified in accordance with the methods of the invention may be administered by gene therapy techniques using genetically modified trophoblasts or by directly introducing genes encoding the inhibitors or stimulators of $TGF\beta_3$, or receptors of cytokines of the TGFβ family, or substances into

trophoblasts *in vivo*. Trophoblasts may be transformed or transfected with a recombinant vector (e.g. retroviral vectors, adenoviral vectors and DNA virus vectors). Genes encoding inhibitors or stimulators, or substances may be introduced into cells of a subject *in vivo* using physical techniques such as microinjection and electroporation or chemical methods such as coprecipitation and incorporation of DNA into liposomes. Antisense molecules may also be introduced *in vivo* using these conventional methods.

The following non-limiting examples are illustrative of the present invention:

EXAMPLE 1

Materials and Methods

Establishment of human trophoblast villous explant culture

Villous explant cultures were established from first trimester human placentae by a modification of the method of Genbacev et al. (21). First trimester human placentae (5-8 weeks gestation) were obtained from elective terminations of pregnancies by dilatation and curettage. Placental tissue was placed in ice-cold phosphate buffered saline (PBS) and processed within two hours of collection. The tissue was washed in sterile PBS, and aseptically dissected using a microscope to remove endometrial tissue and fetal membranes. Small fragments of placental villi (15-20 mg wet weight) were teased apart and placed on a transparent Biopore membrane of 12-mm diameter Millicell-CM culture dish inserts with a pore size of 0.4 µm (Millipore Corp, Bedford, MA). The inserts were precoated with 0.2 ml of undiluted Matrigel (Collaborative Research Inc), polymerized at 37°C for 30 min, and transferred in a 24-well culture dish. Explants were cultured in DMEM/F12 (Gibco, Grand Island, NY) supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin and 0.25 µg/ml ascorbic acid, pH 7.4. Culture media were changed every 48 h and collected for measurement of human chorionic gonadotropin (hCG) and progesterone. Villous explants were kept in culture for up to 6 days. Flattening of the distal end of the villous tips, their adherence to Matrigel and the appearance of extravillous trophoblast cells (EVT) breaking through from the tips, were used as markers of morphological integrity and trophoblast differentiation as previously described by Genbacev et al. (21). EVT cell outgrowth and migration were consistently monitored and quantitated using the ratio of EVT outgrowths/villous tip, where the nominator, EVT outgrowths, represents the number of extravillous trophoblast columns sprouting from the villous tips plus the number of islands of EVT invading into the Matrigel. The denominator represents the total number of villous tips in a single explant culture. EVT outgrowth from the distal end of the villous ups and their migration into the surrounding matrix were observed for up to 6 days in culture.

Initial experiments, in the presence of 10% (v/v) fetal bovine serum (FBS), demonstrated that DMEM/F12 supported greater EVT sprouting and migration than DMEM. In order to study the effect of various agents on EVT differentiation, a serum-free villous explant culture system was developed. Villous explants of 5-8 weeks gestation were incubated overnight in DMEM/F12 or DMEM/F12 + 10% (v/v) FBS to promote attachment of the distal villous tips to the Matrigel. Following this incubation period, explants were washed with fresh medium and cultured in either serum-free DMEM/F12 or DMEM/F12 supplemented with varying concentrations of FBS (0.5% and 10%). In serum-free medium EVT/villous tip was 1.58±0.08 while it was 1.32±0.17 in 0.5% FBS and 1.26±0.02 in 10% FBS

(mean±s.e.m. of 3 separate experiments, each carried out in triplicate), suggesting that villous explant cultures were viable for at least 6 days in a serum-free medium. All subsequent experiments were performed with DMEM/F12 in the absence of serum.

The viability of the explant cultures was assessed by measuring hCG and progesterone production rate in the culture media collected at the time of media change every 48 h. Both hCG and progesterone concentrations were measured by radioimmunoassays (Coat-A-Count HCG IRMA and progesterone; DPC, Los Angeles, CA). Results are expressed for progesterone as ng/0.1 g wet weight tissue and for hCG as IU/0.1g wet weight tissue.

Antibodies

Murine monoclonal antibody (MAb) 44G4 specific for human endoglin was produced as previously described (22). IgG purified from ascites was used in all functional assays. Rat MAb 7D3 against cytokeratin was a gift from Drs. S. Fisher and C. Damsky (San Francisco, CA, USA). Murine MAb TS2/7 against the α_1 integrin subunit was provided by Dr. M. Hemler (Boston, MA, USA). Mouse MAb P1D6 against the α_5 integrin subunit was from Chemicon (Temecula, CA); rat MAb GoH3 against the α_6 integrin subunit was purchased from Serotec Canada (Toronto, Ont. Canada) and the neutralizing rabbit polyclonal antibody to TGF-ß was from R&D (Minneapolis, MN). Purified mouse IgG from Coulter (Hialeah, FL) and rat IgG from Sigma (Diagnostic, Toronto, Ont. Canada) were used as negative controls.

Immunohistochemistry

Villous explants kept in culture for 6 days in the presence or absence of antisense oligonucleotides to endoglin were dissected away from the insert membrane with the supporting Matrigel. Explants and placental tissue of 9 weeks gestation were fixed for 1 h at 4°C in 4% (vol/vol) paraformaldehyde, cryoprotected by incubation in 10% (vol/vol) glycerol for 30 min and 50% (vol/vol) OCT compound (Tissue Tek, Miles, IN) for 18 h, embedded in 100% OCT and frozen in liquid nitrogen. Ten micron sections were cut with a cryostat and mounted on poly-L-lysine coated slides. To verify the quality of the tissue and select the most representative sections, every tenth one was stained with haematoxylin and eosin; neighbouring sections were selected and stained using the avidin-biotin immunoperoxidase method. Endogenous peroxidase enzyme activity was quenched with 3% (vol/vol) hydrogen peroxide in 0.01 M Tris-HCl, pH 7.4, containing 0.15 M NaCl, or methanol for 10 minutes. Non-specific binding sites were blocked using 5% (vol/vol) normal horse serum (NHS) and 1% (wt/vol) BSA in Tris-buffer for 40 min at 23°C. In the case of murine monoclonal antibodies, a higher background was observed and it was necessary to preincubate the sections with 5% (wt/vol) texas red-conjugated goat anti-mouse IgG antibody for 1 h at 23°C prior to incubation with primary antibody at 4° C for 1 h. Optimal antibody concentrations were established in preliminary experiments by titration and were used as follows: 44G4, 5 µg/ml; rabbit anti-TGF-ß, 20 µg/ml; P1D6, 20 µg/ml; GoH3, 0.5 µg/ml; TS2/7, 20 µg/ml; 7D3, 10 µg/ml. The slides were washed three times with Tris-buffer, then incubated with a 200-fold dilution of biotinylated goat anti-rabbit IgG or a 300-fold dilution of biotinylated horse anti-mouse or anti-rat IgG, for 1 h at 4°C. After washing three times with Tris-buffer, the slides were incubated with an avidin-biotin complex for 1 h. Slides were washed again in Tris-buffer

and developed in 0.075% (wt/vol) 3,3-diaminobenzidine in Tris-buffer, pH 7.6, containing 0.002% (vol/vol) H_2O_2 giving rise to a brown product. After light counterstaining with toluidine blue, slides were dehydrated in an ascending ethanol series, cleared in xylene, and mounted. In control experiments, primary antibodies were replaced with non-immune mouse or rat IgG, or blocking solution [5% (vol/vol) NGS and 1% (wt/vol) BSA].

Effect of antibody to endoglin on EVT formation

Villous explants, prepared from placentae of 5-8 weeks gestation, were incubated for 16 h in DMEM/F12. Explant cultures were then washed with fresh serum-free medium and incubated in serum-free DMEM/F12 medium containing increasing concentrations of MAb 44G4 IgG (0.1 to 10 µg/ml). DMEM/F12 medium ± antibody was replaced every 48 h. Antibody addition was thus performed on day 1, 3 and 5 of culture. Morphological integrity of villous explants and their EVT differentiation were monitored daily for up to 6 days.

Antisense oligonucleotides and their effects on EVT formation

Phosphorothioate oligonucleotides (ON) were synthesized on a DNA synthesizer and purified by capillary electrophoresis. Oligonucleotides of 16 base pairs targeted against sequences adjacent to the AUG initiation codon of human endoglin (23) mRNA were synthesized. Previous studies have demonstrated that antisense oligonucleotides, targeted to sequences adjacent to initiation codons, are most efficient in inhibiting translation (24). Furthermore, 16-mer oligonucleotides are short enough to be taken up efficiently and provide sufficient specificity for hybridization to the corresponding target mRNA (24). The sequences of the antisense and sense endoglin oligonucleotides were 5'-GCGTGCCGCGGTCCAT-3' and 5'-ATGGACCGCGGCACGC-3', respectively. An oligomer with the same composition as the antisense oligonucleotide, but with a scrambled sequence, 5'-GCGGGCCTCGTTCCAG-3', was also synthesized and used as a negative control. Oligonucleotides were dissolved in water and their concentration was estimated by optical density at OD_{260}. Antisense or sense oligonucleotides (5-10 µM) were added to the villous explants on day 1 and day 3 of culture. EVT sprouting and migration from the distal end of the villous tips were recorded daily for up to 6 days.

Fibronectin production.

Villous explants of 5-8 weeks gestation were incubated overnight in DMEM/F12. Explants were then washed and incubated in DMEM/F12 containing either 10 µg/ml MAb 44G4 or non-immune IgG, 10 µM antisense, scrambled or sense endoglin oligonucleotides. The medium with or without the various agents was changed on day 3 and was replaced on day 5 by methionine-cysteine free low glucose DMEM containing 25 µCi/ml of [^{35}S]methionine/cysteine with or without the same antibodies or oligonucleotides. The cultures were metabolically labelled for 18 h. Conditioned culture media were collected and diluted with an equal amount of 25 mM Tris-HCl buffer, pH 7.4, 0.15 M NaCl and 0.5% (v/v) Triton X-100 and fibronectin was isolated using gelatin-Sepharose as previously described (25). Briefly, 50 µl of the gelatin-Sepharose suspension was added to 500 µl of medium and the samples were incubated overnight at 4°C. The gelatin-Sepharose beads were centrifuged, washed three times in Tris/Triton X-100 buffer and fibronectin was eluted by boiling for 5 min in 1% (v/v) SDS and

electrophoresed on a 4-12% (w/v) polyacrylamide gradient gels. Radiolabeled fibronectin was revealed by autoradiography and quantitated using a PhosphoImager (410A and Image Quant software, Molecular Dynamics).

[^{3}H]Thymidine incorporation into DNA.

Villous explants of 5-8 weeks gestation, cultured for 48 h with and without antisense ON to endoglin, were incubated in the presence of 1 µCi of [^{3}H]thymidine per milliliter of medium. After 6 h of incubation explants were washed with PBS, fixed in 4% paraformaldehyde for 1 h, embedded in OCT and processed for cryostat sections as previously described. Ten micron sections were mounted on 3-amino-propyl-tryethoxysilane-precoated slides and coated with NBT-2 emulsion (Eastman Kodak, Rochester, NY). Slides were developed after 3 days using Kodak D-19 developer, counterstained with eosin and examined by bright-field microscopy.

Data analysis

All data are presented as means ± s.e.m. of at least three separate experiments carried out in triplicate. Statistical significance was determined by Student's (t)-test for paired groups and by one-way analysis of variance followed by assessment of differences using Student-Newman-Keuls test for non-paired groups. Significance was defined as $p<0.05$.

RESULTS

Stimulation of EVT outgrowth and migration by antibody and antisense oligonucleotides to endoglin

The morphological examination of villous explants of 5-8 weeks gestation, cultured in serum-free medium, revealed a pattern of EVT differentiation (cell outgrowth and migration) similar to that described by Genbacev et al (21). The viability of the explants, as measured by the rate of production of progesterone and hCG, remained relatively constant for up to 6 days.

The ability of an antibody to endoglin (MAb 44G4) to alter the early events of EVT differentiation along the invasive pathway was examined. Exposure of villous explants of 5-8 weeks gestation to 44G4 IgG was associated with an increase in EVT outgrowth from the distal end of the villous tips and a higher number of cells migrating into the surrounding matrix. Stimulation of EVT outgrowth and migration by 44G4 IgG was specific as incubation of explants with an equivalent amount of non-immune murine IgG or medium alone had no effect. Furthermore addition of 44D7 IgG (10µg/ml) reactive with CD98 antigen expressed at high levels on syncytiotrophoblast (26) had no stimulatory effect.

Antisense endoglin also enhanced the number of EVT outgrowths as well as their migration and invasion into the Matrigel. Control explants, cultured in the presence of sense endoglin oligonucleotides, exhibited no such effect.

Further experiments demonstrated that 24 h after the addition of 44G4 IgG (day 2 of culture) there was a significant increase in EVT outgrowth and migration from 0.20 ± 0.03 in the control group to 2.03 ± 0.46 in the antibody treated group ($n=4$; $p< 0.005$). After 5 days of treatment (day 6) the number of EVT outgrowths increased from 0.64 ± 0.09 in control IgG-treated explants to 3.2 ± 0.5 in the 44G4 IgG-treated explants ($n=10$, $p< 0.05$). Subsequent experiments demonstrated that the stimulatory effect of 44G4 IgG was dose-dependent and maximal at 1 µg/ml.

The stimulatory effect of antisense endoglin oligonucleotides on EVT outgrowth and migration was observed on day 3 of culture with 6.87 ± 1.5 in the antisense-treated group versus 1.42 ± 0.41 in the sense-treated group ($p< 0.05$). After 5 days of exposure, the number of EVT/villous tip increased from 2.08 ± 0.47 in sense-treated explants to 8.46 ±1.7 in antisense-treated cultures. The antisense-endoglin effect on trophoblast differentiation was specific as incubation of explants with an equivalent amount of either sense endoglin or scrambled antisense-endoglin oligonucleotide (not shown) had no effect. Antisense endoglin stimulated EVT outgrowth and migration in a concentration-dependent manner with maximal stimulation observed at 10 µM.

Characterization of trophoblast differentiation along the invasive pathway in villous explants culture

Previous reports indicate that stem trophoblasts within the villous core and at the proximal site of the column, where trophoblasts start to migrate away from the stem villi, undergo proliferation (21), whereas differentiated EVT do not. Therefore, studies were carried out to determine if EVT outgrowth triggered by antisense endoglin treatment was due to cell division or migration. [^{3}H]Thymidine autoradiography of explants exposed to antisense endoglin ON showed villous trophoblast proliferation within the villous tip at the proximal site of the forming column, while both differentiated EVT, which have invaded the surrounding Matrigel, and mesenchymal cells in the villous core did not show any DNA synthesis. This suggests that EVT within the column do not divide and that blockage of endoglin most likely induces cell migration from the villous core.

Trophoblast differentiation *in situ* is accompanied by a temporally and spatially regulated switch in integrin repertoire (4). When placental explants of 5-8 weeks gestation were maintained in culture for 5 days in the presence of antisense-endoglin oligonucleotides, the stimulation of EVT outgrowth and migration was also accompanied by changes in integrin expression. The α_6 integrin subunit was found on polarized cytotrophoblasts within the villi and on the non-polarized trophoblasts in the proximal columns. The α_5 integrin subunit was minimally expressed on polarized trophoblasts or syncytium, but was present on EVT within the columns. EVT which had migrated further away in the Matrigel were negative for the α_5 integrin. All trophoblast cells, including CTB within the villi, the syncytiotrophoblast and EVT stained positively for cytokeratin confirming the epithelial-like nature of the cells forming the columns and migrating into the Matrigel. EVT which have migrated into Matrigel were positive for the α_1 integrin. A polyclonal antibody to TGF-ß showed staining of the syncytiotrophoblast and stroma of the villi, suggesting that TGF-ß was present in the culture system. Migrating EVT and the Matrigel itself, known to contain TGF-ß, showed weak positive staining. No reactivity was observed in the explants stained with control IgG.

As little EVT outgrowth is observed under basal culture conditions, the expression of endoglin in trophoblast columns could only be studied in antisense-treated explants. Immunohistochemical analysis of explants treated with antisense oligonucleotides to endoglin revealed that in intact villi the syncytiophoblast maintained high levels of endoglin. Low levels of endoglin and α_5 integrin were observed in the stroma; however this staining appears non-specific as it was also observed with non-immune IgG. The staining of endoglin in EVT of explants treated with antisense endoglin was weakly

positive when compared to sections of the same explant stained with control IgG. In addition, endoglin expression in proximal columns of explants was much reduced when compared to sections of 9 weeks gestation placenta stained under similar conditions. When a subsequent section of this placenta is stained for α_5 integrin, the transition zone in the proximal column is clearly visualized as negative for α_5, but positive for endoglin. The α_5 integrin in explants treated with antisense endoglin was also found to be highly expressed in EVT within proximal and distal columns. These data suggest that antisense endoglin treatment, which promotes EVT outgrowth and migration in explant cultures, induces a decrease in endoglin expression at the level of the transition zone, which is followed by an increase in the expression of the α_5 integrin fibronectin receptor.

Stimulation of fibronectin production by interference with TGF-ß response

FN has been localized to specific regions of the matrix surrounding the anchoring villi and its production is increased during EVT differentiation (27). Thus the effect of either 44G4 IgG or antisense endoglin on fibronectin synthesis by villous explants from 5-8 weeks gestation was investigated. Explants were metabolically labelled on day 4 with [^{35}S]methionine and newly synthesized FN released into the media over a period of 18 h was measured. Both 44G4 IgG and antisense-endoglin oligonucleotides induced a significantly greater production of FN than that observed in control IgG or sense oligonucleotide- treated cultures. PhosphoImager analysis of all data demonstrated a 8- and 5-fold increase in FN synthesis (5 independent experiments carried out in triplicate, $p<0.05$) for 44G4 IgG and antisense-endoglin treated explants, respectively, relative to control sense or DMEM/F12 alone. FN production in villous explants, cultured in the presence of a scrambled antisense endoglin oligonucleotide, was similar to that observed in sense-treated explants or in medium alone.

To demonstrate that endoglin is an essential component of the receptor complex in mediating the effects of TGF-ß$_1$ and TGF-ß$_3$, villous explants were preincubated with either antisense or antibody to endoglin to trigger EVT differentiation. After an overnight incubation, exogenous TGF-ß$_1$, TGF-ß$_2$ or TGF-ß$_3$ were added at a concentration of 10 ng/ml. Explants were metabolically labelled at day 5 of culture and FN synthesis was measured. PhosphoImager analysis demonstrated that both antibody and antisense to endoglin significantly stimulated FN synthesis. Addition of exogenous TGF-ß$_1$ and TGF-ß$_3$ to explant cultures incubated with antisense ON or antibody to endoglin, which binds both isoforms, did not alter the stimulatory effect of antisense ON and antibody to endoglin on FN synthesis. In contrast, addition of TGF-ß$_2$, which does not interact with endoglin, overcame the antibody and antisense ON stimulatory effect on FN synthesis. TGF-ß$_2$, but not -ß$_1$ and -ß$_3$, inhibited also the EVT outgrowth and migration induced by the antisense endoglin treatment.

DISCUSSION

Treatment of human villous explants from 5-8 weeks gestation with antibodies and antisense oligonucleotides to endoglin stimulated EVT differentiation along the invasive pathway. This was manifested by 1) a significant increase in EVT outgrowth and migration, 2) an increase in fibronectin production 3) stem villous trophoblast proliferation and 4) a switch in integrin expression similar to that

observed *in vivo* during formation of anchoring villi. These data suggest that endoglin regulates EVT differentiation during placental development. Endoglin, which is expressed *in vivo* in the transition area where polarized trophoblasts break through the syncytium and begin forming columns of non-polarized cells, appears to be a key molecule in mediating the inhibition of trophoblast differentiation.

During the first trimester of gestation TGF-ß is colocalized with one of its natural inhibitors, decorin, in the ECM of decidual tissue, suggesting that this proteoglycan may aid TGF-β storage or limit its activity within the decidual ECM (12). The findings described herein suggest that TGF-β produced by the villi is a negative regulator of trophoblast differentiation along the invasive pathway. The expression of endoglin at the transitional zone from polarized to non-polarized trophoblasts appears essential to the mediation of this negative regulation. Blocking endoglin expression in this transition phase triggers EVT outgrowth and migration and FN production. Thus, trophoblast invasion, characteristic of normal human placentation, is dependent on an intricate balance between positive and negative regulators. The data herein indicate that endoglin is a critical negative regulator of this system. Therefore, inappropriate expression or function of endoglin may contribute to the major complications of pregnancy such as preeclampsia or choriocarcinoma, associated with abnormal trophoblast invasion and placenta development.

Example 2

The present experiments were conducted to define the precise components that endogenously regulate trophoblast invasion. Using human villous explants of 5-7 weeks gestation it was observed that while trophoblast cells remain viable they do not spontaneously invade into the surrounding matrigel. In contrast, trophoblast cells from 9-13 weeks explants spontaneously invade the matrigel in association with an upregulation of fibronectin synthesis and integrin switching. Trophoblast invasion at 5-7 weeks can be induced by incubation with antisense to TGF-β_3, TGFβ receptor I (ALK-1) or TGFβ receptor II. Only minimal invasion occurred in response to antisense to TGFβ_1 and antisense TGFβ_2 failed to induce invasion. These data suggest that TGF-β_3 via the ALK-1 - receptor II complex is a major regulator of trophoblast invasion *in vitro*. To determine whether this system may also operate *in vivo*, immunohistochemical staining was conducted for TGF-β1 and -3 and for TGFβ receptor I and II in trophoblast tissue from 5-13 weeks of gestation. Strong positive immunoreactivity was observed for TGF-β_3 in both cyto- and syncytiotrophoblast from 5-9 weeks of gestation but immunoreactivity was markedly reduced by 12-13 weeks. Expression of TGFβ_1 was absent at 5 weeks, and transiently expressed at around 8 weeks of gestation. TGF receptor I and II immunoreactivity was strong between 5-8 weeks but was not present at 12-13 weeks. Thus, the presence of TGFβ_3 and its receptors at 5-8 weeks at a time when there is no spontaneous trophoblast invasion *in vitro* and the absence of these molecules at 12-13 weeks when spontaneous in vitro invasion occurs is consistent with a major role for TGF-β_3 as an endogenous inhibitor of trophoblast invasion.

Example 3

Studies were carried out to determine if shallow trophoblast invasion in preeclampsia was associated with an abnormally sustained inhibition of invasion by TGF-β. In particular, the expression/distribution of the different TGF-β isoforms and their receptors was investigated using

immunohistochemical analysis in normal placentae at 7-9 weeks (at the onset of trophoblast invasion) at 12-13 weeks (the period of peak invasion), in control placentae between 29 and 34 weeks and in preeclamptic placentae ranging from 27 to 34 weeks. In normal placentae, TGF-β_3 expression was markedly reduced with advancing gestational age. Expression was high in cyto- and syncytiotrophoblast cells at 7-9 weeks of gestation but was absent in villous tissue at 12-13 weeks and at 29-34 weeks of gestation. A similar decline in positive immunoreactivity against TGF-β receptor I and II was also observed over this time period. In contrast, in preeclamptic placentae between 27-34 weeks of gestation, strong staining for TGF-β_3 and its receptors was present in syncytiotrophoblast and stromal cells. immunopositive reactivity was not detected against TGF-β_1 or TGF-β_2 in either normal or preeclamptic placentae. These data indicates that preeclampsia may result from a failure of trophoblast cells to downregulate expression of TGF-β_3 and its receptors which continue to exert an inhibitory influence on trophoblast invasion into the uterine wall.

Example 4

Materials and Methods:

RT-PCR and Southern Blot Analysis

Total RNA was extracted from the placenta, reverse transcribed and amplified by 15 cycles of PCR using TGFβ isoform specific primers. RT-PCR products were analysed by Southern blotting using ^{32}P-labelled TGFβ cDNAs. The primer set chosen for amplification of TGFβs were based on human mRNA sequences. Primers used for amplification were: (a) TGFβ$_1$ cDNA: (forward primer): 5'-GCCCTGGACACCAACTATTGCT-3', (reversed primer): 5'-AGGCTCCAAATGTAGGGGC AGG-3' (predicted product size = 161 bp); (b) TGFβ$_2$ cDNA (forward primer): 5'-CATCTGGTCCCGGTGGCGCT-3', (reversed primer): 5'-GACGATTCTGAAGTAGGG-3' (predicted product size = 353 bp); (c) TGFβ$_3$ cDNA: (forward primer): 5'-CAAAGGGCTCTGGTGGTCCTG-3', (reversed primer): 5'-CTTAGAGGTAATTCCCTTGGGG-3' (predicted product size = 374 bp); (c) β-acun cDNA: (forward primer): 5'-CTTCTACAATGAGCTGGGTG-3', (reversed primer): 5'-TCATGAGGTAGTCAGTCAGG-3' (predicted product size = 307 bp). The identity of the PCR reaction products was also confirmed by sequencing.

Immunohistochemistry

Placental tissue was processed for immunocytochemistry as previously described (I. Caniggia et al *Endocrinology*. **138**, 3976 1997). Purified rabbit polyclonal antibody directed against TGFß$_1$, TGFß$_2$ and TGFß$_3$ (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:50 dilution. Sections (7μm) were stained using the avidin-biotin immunoperoxidase method (I. Caniggia et al *Endocrinology*. **138**, 3976 1997). Control experiments included replacement of primary antibodies with antiserum preincubated with an excess of TGFβs (competing peptide) or with blocking solution [5% (vol/vol) NGS and 1% (wt/vol) BSA].

Human Villus Explant Culture System

Villous explant cultures were established as described previously (I. Caniggia et al *Endocrinology*. **138**, 3976 1997, O.Genbacev et al., Placenta 13:439, 1992) from first trimester human

placentae (5-10 weeks gestation) or from preeclamptic and age-matched control placentae (30 and 32 weeks of gestation) after collection according to ethical guidelines. The preeclamptic group was selected according to both clinical and pathological criteria (L. Chesley, *Obstet. Gynecol.* **65**, 423, 1985). Following an overnight period in serum-free DMEM/F12, explants were cultured in media containing antisense or sense oligonucleotides (10μM) for up to 6 days (with changes of media/oligonucleotides every 48 hours). Phosphorothioate oligonucleotides of 16 base pairs targeted against sequences adjacent to the AUG initiation codon of different human TGFβ isoforms mRNA were synthesized as follows: $TGF\beta_1$ 5'-CCCCGAGGGCGGCATG-3' and 5'-CATGCCGCCCTCGGGG-3', respectively; $TGF\beta_2$ 5'-CACACAGTAGTGCATG-3' and 5'-CATGCACTACTGTGTG-3'; $TGF\beta_3$ 5'-CCTTTGCAAGTGCATC-3' and 5'-GATGCACTTGCAAAGG-3'.

Fibronectin Synthesis

To measure fibronectin synthesis on day 5 explants were cultured in the presence of 25 μCi/ml of [^{35}S]methionine/cysteine for 18 hours. Conditioned culture media were collected and diluted with an equal amount of 25 mM Tris-HCl buffer, pH 7.4, 0.15 M NaCl and 0.5% (v/v) Triton X-100 and fibronectin was isolated using gelatin-Sepharose as previously described (I. Caniggia et al *Endocrinology*. **138**, 3976 1997, E. Engvall et al *Int. J. Cancer*. **20**: 1, 1977). Radiolabeled fibronectin was revealed by autoradiography and quantitated using a PhosphoImager (410A and Image Quant software, Molecular Dynamics).

Gelatinolytic Activity

Analysis of gelatinolytic activity was performed using 10% polyacrylamide gel (wt/vol) impregnated with 0.1% gelatin (NOVEX, San Diego, CA) as previously described (I. Caniggia et al *Endocrinology*. **138**, 3976 1997). For Western blot analysis of metalloproteases expression, 5 μl of conditioned media were subjected to gel electrophoresis using 10% polyacrylamide gels. Proteins were then blotted to Westran PVDF membrane. Primary antibodies were used at 1:100 dilution, and detected using horse radish peroxidase conjugated antimouse IgG (Amerham 1:10.000 fold dilution) and enhanced by chemiluminescence (ECL, Amerham).

Results:

The expression of TGFβ around 9-12 weeks of pregnancy and its relationship to trophoblast invasion and subsequently preeclampsia were investigated. Using low cycle RT-PCR followed by Southern blot analysis all three isoforms of TGFβ were found to be expressed during the first trimester (Figure 3A). However, while transcripts corresponding to $TGF\beta_1$ and $TGF\beta_2$ were uniformly expressed throughout this period, the expression of $TGF\beta_3$ exhibited a striking pattern of developmental or temporal regulation. $TGF\beta_3$ mRNA levels were relatively low at 5-6 weeks, increased markedly between 7 and 8 weeks, and then fell precipitously at 9 weeks. This pattern of expression for the $TGF\beta_3$ isoform was confirmed at the protein level by immunohistochemistry (Figure 3B). $TGF\beta_3$ was localized to cyto and syncytiotrophoblasts within the villous and also to cytotrophoblasts within the invading column (Figure 3B). $TGF\beta_3$ was noticeably absent in those cytotrophoblast cells at the transition between polarized and non-polarized cells at the proximal site of the forming column. Importantly, the down-

regulation of TGFβ$_3$ around 9 weeks is temporally associated with the period of maximal trophoblast invasion *in vivo* and the expression of markers of cytotrophoblast invasion, including switching of integrin isoforms (Damsky, C. H. et al Development 120:3657, 1994), synthesis of matrix ligands for these integrins (P. Bischof, L. Haenggeli A. Campana, *Human Reprod.* **10**, 734. (1995), M. J. Kupferminc, A. M. Peaceman, T. R. Wigton, K. A. Rehnberg, M. A. Socol, *Am.J. Obstet. Gynecol.* **172**, 649 (1995)) and upregulation of gelatinase A (MMP2) and gelatinase B (MMP9) activity (C. I. Librach, et al. *J.Biol. Chem.* **269**, 17125. (1994)).

To determine the functional significance of the TGFβ expression patterns, a human villus explant culture system was used which mimics closely the normal pattern of trophoblast invasion *in vivo* (I. Caniggia, C. V. Taylor, J. W. K. Ritchie, S. J. Lye, M. Letarte, *Endocrinology*. **138**, 4977 (1997), O. Genbacev, S.A. Schubach, R.K. Miller, *Placenta* **13**, 439. (1992)). Morphologic (EVT outgrowth) and biochemical (fibronecun [FN] synthesis and gelatinase activity) indices of trophoblast invasion were monitored in response to antisense (AS) induced suppression of TGFβ isoform expression in explants at 5-8 weeks of gestation. Explants exposed to AS TGFβ$_3$ (but not TGFβ$_1$ or TGFβ$_2$) displayed prominent EVT outgrowth from the distal end of the villous tip (Figure 4A). This morphologic response was associated with a significant increase in FN synthesis (Figure 4B, and Figure 4E) and gelatinase activity (Figure 4D). The specificity of the AS TGFβ$_3$ response was demonstrated by reversal of both morphologic and biochemical indices when AS-treated explants were cultured in the presence of TGFβ$_3$ but not TGFβ$_1$ (Figure 4C). The induction of FN synthesis by AS TGFβ$_3$ at 5-8 weeks was lost at 9-13 weeks (Figure 4E) further demonstrating the specificity of the AS action as TGFβ$_3$ is not expressed in villous trophoblast at 9-12 weeks.

These functional data together with the temporal-spatial expression patterns strongly suggest that down-regulation of TGFβ$_3$ around 9-12 weeks is required for optimal trophoblast invasion indicate that a failure to down-regulate TGFβ$_3$ expression is the basis of limited trophoblast invasion found in preeclampsia. Significantly higher levels of mRNA encoding TGFβ$_3$ (but not TGFβ$_1$ or TGFβ$_2$) were found in preeclamptic versus control placentae (Figure 5A). Immunoreactive TGFβ$_3$ intensively labelled syncytio and cytotrophoblasts in villous tissues from preeclamptic patients while little or no immunoreactivity was present in the age-matched controls (Figure 5B). Elevated levels of FN mRNA and a failure to complete integrin switching (i.e the trophoblast remain positive for α_5 and fail to express α_1 were also obverved in preeclamptic placentae. These data suggest that the trophoblasts from preeclamptic placenta are arrested at a relatively immature phenotype possibly due to a failure to undergo complete differentiation along the invasive pathway during the first trimester of gestation.

To determine whether there was functional significance associated with overexpression of TGFβ$_3$ in preeclamptic placentae, the pattern of trophoblast differenuation along the invasive pathway in explants from control and preeclamptic patients was analyzed. When cultured on matrigel, explants from non-preeclamptic patients showed formation of EVT columns which spontaneously invaded into the surrounding matrigel. In contrast, explants from preeclamptic placentae failed to exhibit EVT outgrowth or invasion (Figure 6A). These data are consistent with the view that preeclampsia is associated with reduced invasive capability of trophoblasts. Of critical importance to the investigation was whether this

reduced invasive capability was due to the overexpression of $TGF\beta_3$. Therefore the differentiation of villous explants from preeclamptic patients cultured in the presence of AS $TGF\beta_3$ was monitored. In contrast to untreated or sense-treated controls, treatment of explants from preeclamptic patients with AS $TGF\beta_3$ restored the invasive capability, as demonstrated by the formation of EVT columns migrating through the matrigel (Figure 6A). The invasive nature of this phenotype was confirmed by the finding that explants treated with AS $TGF\beta_3$ acquired the expression of gelatinase B/MMP9, an enzyme which is normally only expressed in trophoblast cells that are highly invasive (Figure 6B and Figure 6C).

The data presented here demonstrate not only that abnormalities in $TGF\beta_3$ expression are associated with preeclampsia but also that down-regulation of $TGF\beta_3$ with antisense oligonucleotides restores the invasive capability of preeclamptic trophoblasts. The data are consistent with a model of normal placentation in which down-regulation of $TGF\beta_3$ expression in trophoblast around 9 weeks of pregnancy permits differentiation of trophoblast to EVT that form the anchoring villi and from which derive the α1-integrin positive EVT which invade deep into the maternal uterus. This invasion contributes to the remodelling of the uterine spiral arteries and ultimately enables the establishment of increased vascular perfusion of the placenta. In placentae predisposed to preeclampsia, $TGF\beta_3$ expression remains abnormally elevated and trophoblasts remain in a relatively immature state of differentiation. As a direct consequence, trophoblast invasion into the uterus is limited and uteroplacental perfusion is reduced. This conclusion is consistent with the clinical manifestations of preeclampsia, including shallow trophoblast invasion into the uterus and abnormally high uteroplacental vascular resistance.

Example 5

Role of O_2 Tension in Trophoblast Invasion

The role of oxygen tension in regulating trophoblast differentiation along the invasive pathway has been investigated. The data indicate that expression of hypoxia inducible factor HIF-1α parallels that of $TGF\beta_3$ in first trimester trophoblast (i.e. peaks at 6-8 weeks but decreases after 9-10 weeks when oxygen tension increases (Figure 7A). The presence of putative HIF-1 binding sites in the promoter region of the $TGF\beta_3$ gene suggests that induction of HIF-1α by low PO_2 (around 6-8 weeks) up regulates $TGF\beta_3$ transcription and blocks a trophoblast invasion. A failure of the system to down-regulate at 9-12 weeks (either due to a block in response to normoxia or the absence of an increase in oxygen tension) could lead to shallow invasion and predispose to preeclampsia. This is supported by data showing that expression of HIF-1α is dramatically increased in placentas of preeclamptic patients when compared to age-matched control tissue (Figure 7B). In Figure 7A and 7B mRNA HIF-1α expression was assessed by using low cycle RT-PCR followed by Southern blot analysis. This is also supported by Figure 8 showing the effect of low oxygen tension of $TGF\beta_3$ and HIF-1α expression in villous explants; Figure 9 showing the effect of low oxygen tension on villous explant morphology; and Figure 10 showing the effect of antisense to HIF-1α on villous explant morphology.

Example 6

$TGF\beta_3$ Signals Through a Receptor Complex

In addition to endoglin, evidence indicates that $TGF\beta_3$ signals through a receptor complex which includes RI (ALK-1) and RII. While TGFβ R-I (ALK-5) and TGFβ R-II are expressed throughout the villi

and decidua at 9-10 weeks gestation; they are absent from the base of the proximal columns of the anchoring villi at the transition zone between the villous and the invading EVT, exactly at the site where endoglin is upregulated. This dramatic change in TGF-β receptor expression suggests that EVTs within the columns *in situ* are not subject to the inhibitory actions of TGFβ but via R-I and R-II they do come under the control of this ligand upon entering the decidua. The potential clinical importance of the TGFβ receptor system in trophoblast invasion is highlighted by data demonstrating that beside $TGF\beta_3$, R-I is expressed at greater levels in trophoblast tissue of preeclamptic patients when compared to that in age-matched control placenta. Antisense disruption of R-I (ALK-1) and R-II expression stimulated trophoblast outgrowth/migration and FN synthesis. In contrast, antisense to R-I (ALK-5) inhibited FN synthesis.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Below full citations are set out for the references referred to in the specification and detailed legends for the figures are provided.

FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

1. Cross JC, Werb Z, Fisher, SJ. 1994. Implantation and the placenta: key pieces of the development puzzle. Science. 266: 1508-1518.

2. Zhou Y, Damsky C H, Chiu K, Roberts JM, Fisher SJ. 1993. Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts. J. Clin. Invest. 91: 950-960.

3. Aplin JD. 1991. Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro. J. Cell Science. 99: 681-692.

4. Damsky CH, Fitzgerald ML, Fisher SJ. 1992. Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. J. Clin. Invest. 89: 210-222.

5. Bischof P, Redard M, Gindre P, Vassilakos P, Campana A. 1993. Localization of alpha2, alpha5 and alpha6 integrin subunits in human endometrium, decidua and trophoblast. Eur. J. of Obst. and Gyn. and Repr. Biol. 51: 217-226.

6. St-Jacques S, Forte M, Lye SJ, Letarte M. 1994. Localization of endoglin, a transforming growth-factor-b binding protein, and of CD44 and integrins in placenta during the first trimester of pregnancy. Biol Reprod. 51: 405-413.

7. Fisher SJ, Cui T, Zhang L et al. 1989. Adhesive and degradative properties of human placental cytotrophoblast cell in vitro. J. Cell. Biol. 109: 891-902.

8. Librach CL, Feigenbaun SL, Bass KE, et al. 1994. Interleukin-1b regulates human cytotrophoblast metalloproteinase activity and invasion in vitro. J. Biol. Chem. 269: 17125-17131.

9. Bass K E, Morrish D, Roth I, et al. 1994 Human cytotrophoblast invasion is up-regulated by epidermal growth factor: evidence that paracrine factors modify this process. Devel. Biol. 164: 550-561.

10. Graham CH, Lysiak JJ, McCrae KR, Lala PK. 1992. Localization of transforming growth factor-b at the human fetal-maternal interface: role in trophoblast growth and differentiation. Biol. Reprod. 46: 561-572.

11. Graham CH, Lala PK. 1991. Mechanism of control of trophoblast invasion in situ. J. Cell. Physiol. 148: 228-234.

12. Lysiak JJ, Hunt J, Pringle JA, Lala PK. 1995. Localization of transforming growth factor b and its natural inhibitor decorin in the human placenta and decidua throughout gestation. Placenta. 16: 221-231.

13. Irving JA, and Lala PK. 1995. Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-b, IGF-II, and IGBP-1. Exp. Cell. Res. 217: 419-427.

14. Cheifetz S, Bellon T, Cales C, et al. 1992. Endoglin is a component of the transforming growth factor-b receptor system in human endothelial cells. J. Biol. Chem. 267: 19027-19030.

15. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J. 1994. Mechanisms of activation of the TGF-b receptor. Nature. 370: 341-347.

16. Mitchell EJ, Fitz-Gibbon L, O'Connor-McCourt MD. 1992. Subtypes of betaglycan and type I and type II transforming growth factor-b (TGF-b) receptors with different affinities for TGF-b1 and TGF-b2 are exhibited by human placenta trophoblasts. J. Cell. Physiol. 150: 334-343.

17. Gougos A, St-Jacques S, Greaves A, et al. 1992. Identification of distinct epitopes of endoglin, an RGD-containing glycoprotein of endothelial cells, leukemic cells, and syncytiotrophoblasts. Int. Immunol. 4: 83-92.

18. Yamashita H, Ichijo H, Grimsby S, Moren A, ten DijkeP, and Miyazono K. 1994. Endoglin forms an heteromeric complex with the signalling receptors for transforming growth factor-b. J. Biol. Chem. 269: 1995-2001.

19. Zhang H, Shaw ARE, Mak A, and Letarte M. 1996. Endoglin is a component of the TGF-b receptor complex of human pre-B leukemic cells. J. Immunol. 156: 565-573.

20. Lastres P, Letamendia A, Zhang H, et al. 1996. Endoglin modulates cellular responses to TGF-b1. J. Cell Biol.133: 1109-1121.

21. Genbacev O, Schubach SA, Miller RK. (1992). Villous culture of first trimester human placenta - Model to study extravillous trophoblast (EVT) differentiation. Placenta. 13: 439-461.

22. Quackenbush EJ and Letarte M. 1985. Identification of several cell surface proteins of non-T, non-B acute lymphoblastic leukemia by using monoclonal antibodies. J. Immunol. 134: 1276-1285.

23. Gougos A, and Letarte M. 1990. Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. J. Biol. Chem. 265: 8361-8364.

24. Malcolm ADB. 1992. Uses and applications of antisense oligonucleotides: uses of antisense nucleic acids-an introduction. Bioch. Soc. Trans. 20: 745-746.

25. Engvall E, and Ruoslahti E. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int. J. Cancer. 20: 1-5.

26. Soubiran P, Hsi B-L, Lipinski M, Yeh C-J G, Vaigot P, Masseyeff R. 1986. Distribution of Trop 3 and 4 antigens as defined by monoclonal antibodies raised against a human choriocarcinoma cell line. A.J.R.I.M. 12: 118-123.

27. Feinberg RF, Kilman HJ, Locwood CJ. 1991 Is oncofetal fibronectin a trophoblast glue for human implantation? Am. J. Pathol. 138: 537-543.

28. Vicovac I, Jones CJ, Aplin JD. 1995. Trophoblast differentiation during formation of anchoring villi in a model of the early human placenta in vitro. Placenta. 16: 41-56.

29. Feinberg RF, Kliman HJ, Wang C-L. 1994. Transforming growth factor-b stimulates trophoblast oncofetal fibronectin synthesis in vitro: implications for trophoblast implantation in vivo. J.Clin. Endocrinol Metab. 78: 1241-1248.

30. Bischof P, Haenggeli L, and Campana A. 1995. Gelatinase and oncofetal fibronectin secretion is dependent on integrin expression on human cytotrophoblasts. Molecular Human Reproduction. 10: 734-742.

31. Damsky CH, Librach C, Lim K-H, et al. 1994. Integrin switching regulates normal trophoblast invasion. Development. 120: 3657-3666.

DETAILED FIGURE LEGENDS

Figure 3 Expression of TGF-ß isoforms in human placenta in the first trimester of gestation. (Figure 3A) Message expression of TGFß isoforms was assessed by low cycle RT-PCR followed by Southern blot analysis using specific probes for $TGF\beta_1$, $TGF\beta_2$ and $TGF\beta_3$ and the control house-keeping gene ß-actin. Note that $TGF\beta_3$ expression increases around 7-8 weeks gestation and declines thereafter. (Figure 3B) Immunoperoxidase staining of $TGF\beta_3$ was performed in placental sections at 5, 8 and 12 weeks of gestation. Sections of placental tissue of 5 weeks gestation show positive immunoreactivity visualized by brown staining in the cytotrophoblast, syncytiotrophoblast and stromal cells of the chorionic villi. Sections of placental tissue of 8 weeks gestation show strong positive immunoreactivity in the cytotrophoblast, syncytiotrophoblast, and stromal cells. Note that TGF-$ß_3$ was expressed in the non-polarized trophoblast within the column (EVT, thin arrow) but was absent in the transitional zone where polarized cells become unpolarized (thick arrows). Sections of placenta at 12 weeks gestation demonstrate low or absent TGF-$ß_3$ immunoreactivity in the villi. There is no immunoreactivity when antiserum was preincubated with an excess of $TGF\beta_3$ competing peptide (8 weeks, control).

Figure 4 Antisense $TGF\beta_3$ stimulates trophoblast migration, fibronectin production and gelatinase activity. Explants of 5-8 weeks gestation were treated for 5 days with 10 µM antisense oligonucleotides to $TGF\beta_3$ (AS-ß3), AS-ß3 plus 10 ng/ml recombinant $TGF\beta_3$ (AS-ß3+ß3) or AS-ß3 plus recombinant TGFß1 (AS-ß3+ß1). Control experiments were run in parallel using sense TGF-$ß_3$ (S-ß3) or medium alone (Figure 4C). (Figure 4A) Shown is a representative experiment demonstrating that addition of recombinant $TGF\beta_3$ to antisense $TGF\beta_3$ treated explants (AS-ß3+ß3) abolishes the antisense stimulatory effect on trophoblasts budding and outgrowth (arrows). (Figure 4B) Similar reversal effect on AS-ß3 stimulatory effect by exogenous $TGF\beta_3$ was demonstrated also for fibronectin synthesis. Representative analysis of triplicate samples from a single experiments is shown. The position of the marker with $M_r = 200x10^3$ is indicated. Lanes 1-3, S-ß3 treated explants; lanes 4-6, AS-ß3 treated explants; lanes 7-9, AS-ß3+ß3 treated explants. (Figure 4C) Changes in fibronectin estimated after normalization to control cultures. Antisense $TGF\beta_3$ treatment (AS-ß3, solid bar) significantly increased ($p<0.05$; one-way ANOVA followed by Student-Newman-Keuls test for non-paired groups) the amount of labelled fibronectin compared to both medium alone (Figure 4C, open bar) or sense (S-ß3, cross bar). Addition of exogenous $TGF\beta_3$ (AS-ß3+ß3 squares bar) but not $TGF\beta_1$ (AS-ß3+ß1 cross hatched bar) to the antisense treated explants abolished the antisense stimulatory effect on fibronectin production, demonstrating the specificity of the action of $TGF\beta_3$. (Figure 4D) Gelatinase activity in conditioned media of explants treated with sense or antisense oligonucleotides to TGFß3. Arrows indicate positions of gelatinases activity (MMP2: 60, 68; MMP9: 84 and 92, kDa). (Figure 4E) The antisense $TGF\beta_3$ stimulatory effect on fibronectin production is lost after 9 weeks of gestation. Explants of 6 and 10 weeks gestation were treated with 10 µM antisense (AS-ß3) or control sense (S-ß3) oligonucleotides to $TGF\beta_3$. Newly synthesized fibronectin was isolated from the medium as described above. Representative analysis of

triplicate samples from a single experiment is shown. Lanes 1-3 and 7-9, S-ß3 treated explants; lanes 4-6 and 10-12, AS-ß3 treated explants.

Figure 5 $TGF\beta_3$ is overexpressed in preeclamptic placentae. (Figure 5A) Message expression of TGFß isoforms, α_5 integrin receptor and fibronectin in preeclamptic (PE) and age-matched control placentae (Figure 5C) was assessed by low cycle RT-PCR followed by Southern blot analysis using specific probes for $TGF\beta_1$, $TGF\beta_2$, $TGF\beta_3$, α_5, fibronectin and the control house-keeping gene ß-actin. Note that $TGF\beta_3$, α_5 and fibronectin, but not $TGF\beta_1$ or $TGF\beta_2$, expression were higher in preeclamptic placentae when compared to age-matched control. (Figure 5B) Immunoperoxidase staining of $TGF\beta_3$ was performed in placental sections from normal pregnancies and pregnancies complicated by preeclampsia. Sections of normal placental tissue of 29, 31 and 33 weeks of gestation show low/absent $TGF\beta_3$ immunoreactivity in cells of the chorionic villi. Sections of preeclamptic placental tissue of the same gestation show strong positive immunoreactivity visualized by brown staining in the cytotrophoblast, syncytiotrophoblast and stromal cells of the chorionic villi. Control experiments were performed using antiserum preabsorbed with an excess of peptide.

Figure 6A Antisense oligonucleotides to $TGF\beta_3$ induces the formation of columns of trophoblast cells in preeclamptic villous explants. Villous explant cultures were prepared from preeclamptic and age-matched control placentae. Explants were mantained in culture in the presence of either control sense or antisense oligonucleotides to $TGF\beta_3$ for 5 days. Morphological integrity was recorded daily. Explants from normal placenta (32 weeks), exposed to sense oligonucleotides (S-ß3) spontaneously form columns of trophoblast cells which migrate and invade into the surrounding Matrigel (arrows), while explants from preeclamptic placenta (32 weeks) exposed to sense oligonucleotides do not. In contrast, antisense treatment (AS-ß3) triggers the formation of invading trophoblast columns (arrows) in preeclamptic placentae.

Figure 6B and Figure 6C. Antisense oligonucleotides to $TGF\beta_3$ triggers gelatinase activity and expression in preeclamptic villous explants. Explants of 32 weeks gestation from preeclamptic placentae were treated with antisense (AS-ß3) or control sense (S-ß3) oligonucleotides to $TGF\beta_3$ for 5 days. Samples of conditioned medium were collected at day 5 and subjected to analysis by gelatin Zymography (Figure 6B) or Western blotting with MMP9 antisera (Figure 6C). Arrows indicate positions of gelatinases activity (MMP-2: 60, 68; MMP-9: 84 and 92, kDa).